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IMMOBILIZATION OF ENZYMES BY CROSS-LINKING WITH A CROSS-LINKING AGENT AND A POLYMER CONTAINING 1-AMINO ETHYLENE MOIETIES

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Abstract: A polymer containing 1-amino ethylene moieties and, optionally, N-vinyl formamide is used in immobilization of enzymes together with a cross-linking agent such as glutaraldehyde. Physically strong particles suited for use (^) in a fixed-bed column are obtained.

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Detailed Description

I 01 IMMOBILIZATION OF ENZYMES BY CROSS-LINKING WITH A CROSS-LINKING AGENT AND A POLYMER CONTAINING L-AMINO ETHYLENE MOIETIES.

TECHNICAL FIELD This invention relates to a particulate immobilized enzyme preparation, to use of the immobilized enzyme preparation in an enzyme-catalyzed process and to a process for immobilizing enzymatically active biological material.

BACKGROUND ART It is known that enzymes can be immobilized without using a carrier by cross-

linking with polyethylene imine (PEI) and a cross-linking agent such as glutaralclehyde or polyazetidine (e.g. US 4,288,552, US 4,355,105, EP 297,912). Such methods can be used to produce immobilized enzymes in particle form with good activity and physical strength, suitable for continuous use in a fixed-bed column. However, PEI is an expensive material, so it is desirable to find alternatives to this. Also, immobilization of some enzymes with PEI some times leads to poor flocculation, resulting in difficult dewatering.

It is the purpose of the invention to provide a method with improved flocculation and dewatering and without the need for PEI for producing an immobilized enzyme preparation with satisfactory properties for fixed- bed column use: activity, stability (half life) and physical strength (pressure drop).

SUMMARY OF THE INVENTION It has surprisingly been found that the object can be obtained by replacing polyethylenei mine with a certain polymer that has never been described for use in immobilization.

- I 1~ Accordingly, the invention provides a particulate immobilized enzyme preparation obtainable by a process comprising the sequential steps of:
- a) providing an aqueous medium containing enzymatically active biological material b) adding a polymer containing 1 -amino ethylene moieties and, optionally, N-vinyl formamide, c) adding a cross-linking agent for amino groups, d) holding the mixture to effect cross-linking and flocculation, e) dewatering, f) sub-dividing, and g) drying.

The invention also provides use of the immobilized enzyme preparation in an enzyme-catalyzed process. Finally, the invention provides a process for immobilizing enzymatically active biological material, characterized by comprising the above sequential steps.

DETAILED DESCRIPTION OF THE INVENTION The biological material to be immobilized according to the invention is enzymatically active. It may comprise enzymatically active microbial cells in the form of a culture broth containing intact cells or cell paste consisting of partly or fully disrupted cells. The biological material may also consist of or comprise a cell-free enzyme solution or purified enzyme.

The biological material may also comprise inactive protein, preferably 0-50% by weight. Thus, if highly purified enzyme is to be immobilized, it may be preferable to add inert protein such as albumin or gelatin.

The quantity of water present in the reaction mixture is not critical.

Excess water will be removed during clewatering without any serious loss of active material. Thus, water may be added to obtain a convenient consistency.

Conveniently, the biological material is added in the form of an aqueous dispersion or solution typically with 1-25% (w/w) of dry substance, particularly 1-10% in the case of culture broth or 10-25% in the cae of a purified enzyme.

The polymer to be used in the invention may be a homopolymer of 1 - aminoethylene or a copolymer of this monomer and N-vinyl formamide. It preferably contains 10-100 mole % (most preferably 25-50%) of -CH2- CH(NH2)- units and 0-90% mole % (most preferably 50-75%) of -CH2-CH(NH-CHO)- units. It preferably has a molecular weight in the range 50,000-500,000. Such polymers may be produced by hydrolysis of N-vinyl formamide homopolymer, e.g. according to US 4,421,602 or EP 71,050. The molecular weight of the polymer is described in this application by the Fikentscher K value of the non-hydrolysed N- vinyl formamide homopolymer as descNbed in US Q21502. This K value can vary

between 10 and 200. The amount of polymer is preferably 2-30% by weight of the dry matter in the biological material and most preferably 2-15%.

Chitosan may be used in addition to the above-mentioned polymer.

Preferably, 1-15% of chitosan and 1-15% of the polymer are used (% by weight of the dry matter in the biological material). The chitosan should be introduced before the addition of cross-linking in step c); it may be added together with the polymer.

The cross-linking agent used in the invention is one that reacts with amino groups. Examples are glutaraldehyde, diisocyanates, (e.g. toluylene or hexamethylene diisocyanate) and polyazetidine (as described in EP 297, 912).

Optionally, cross-linking agent may also be added to the aqueous medium of step a) and the mixture held sufficiently long to let partial cross-linking occur (e.g. 5-20 minutes) befor e introducing the polymer. In this case, the ratio of cross-linking agent added in step a) to that added in step c) is preferably 1:2 - 1:4.

The total amount of cross-linking agent is preferably in the range 5-% by weight of the dry matter in the biological material. A relatively high amount, e.g. 20-40%, may be preferred in order to obtain physically strong particles; alternatively, a lower amount (e.g. 10-20%) may be used in order to obtain particles with less diffusion restriction and higher activity. The holding time in step d) is preferably 0.5 - 2 hours.

The temperature throughout the process is generally in the range 0-60*C. Temperature near ambient is often convenient, but lower temperature may be needed due to enzyme instability.

pH throughout the process is generally around neutral, mostly between about 5 and about 9. Higher or lower pH may be preferred depending on the enzyme stability. A buffer may be included to stabilize pH during the reaction.

The dewatering step of the invention is intended to remove excess water after flocculation thereby generating a pasty mass. It is conveniently done by filtration or centrifugation.

Sub-dividing according to the invention is done to form the dewatered mass into individual particles of controlled size. A preferred technique is extrusion.

Optionally, the extruded particles may be rounded (spheronized) before or after curing, e.g. by the "Marumerizer" technique disclosed in British patent specification GB 1,362,265.

The subdivided material is dried, e.g. to a water content below of 10- % w/w. With an ultimate water content above 25% the microbial stability of the product may be unsatisfactory, and the particles may tend to aggregate over time in storage. Drying to a water content of below about 10% may inactivate the biological material. Preferred techniques are air drying or fluidized- bed drying, generally at 15-80"C. In case of very sensitive biological materials, low tempera- ture drying or freeze-drying may be needed.

The invention may be applied to immobilization of a wide range of enzymes. Some examples follow:

- Glucose isomerase, e.g. derived from Streptomyces (especially S. murinus), Bacillus (especially B. coagulans) or Actingolanes (especially A. missouriensis).
- Arninopepticlase, e.g. derived from Pseudomonas

- Penicillin acylase, e.g. derived from Fusarium.
- Nitrilase, e.g. from Rhodococcus (especially Rh. erythropolis), from Reudomonas or from Brevibacterium.
- Fructosyl transferase, e.g. from Aspergillus.
- Invertase, e.g. from Saccharomyces. Lactas% mg. from Kluyveromyces.
- Cyaniclase, e.g. from Alcaligenes. EXAMPLES B(AMPLE 1 A reference sample was prepared as follows:

1 1 of glucose isomerase containing fermentation liquid from Streptomyces murinus (prepared according to US 4,687,742, dry substance content 4%), was mixed with 10 g MgSO,.7H,O and pH adjusted to 7.5. 5 ml 50% glutaralclehyde was added and the cell sludge was stirred for 10 minutes under pH-adjustment to pH 7.5. Then 5.0% (on dry matter) of pol yethylene imine (Sedipur, product of BASF, West Germany) was added and after thorough mixing ml glutaraldehyde (total glutaralclehyde 18% based on cell sludge plus poly- ethylene imine dry substance) for cross-linking of the mixture. pH was constantly adjusted to 7.5. After 1 hour the cross-linked mixture was flocculated by addftion of a cationic flocculant, Superfloc C 521 (Cyanamid Int.). The -cross- linked enzyme was recovered by filtration, formed into particles by extrusion through a 0.8 mm screen and dried at room temperature.

A series of preparations according to the invention were made as described above, but with polyethyleneinine repaced by a polymer of 1 - amino ethylene with or without N-vinyl formamide (experimental preparations from BASF, Germany). The composition of the polymers is shown in Table 1

ITC)91/08287 PCr/DK90/00305 The glucose isomerase activity was measured by Novo Analysis Method F-85531 0 (available on request from Novo Nordisk A/S, Denmark). Both initial IL activity of the immobilized enzyme and in some cases the activity during several months of continuous isomerization in laboratory scale columns (60 0 C, pH 7.5) was measured. The activity decay is expressed by the half life, the time where the activity is equal to half of the initial activity.

The pressure drop was measured over a column with a diameter of 24 mm and an enzyme bed height of 4 cm (5 g enzyme). The solution, 45% glucose in demineralized water with 1 g MgSO,/I, was pumped through the column at a rate of 40 g/min at 60"C. The pressure drop (in mm of liquid) describes the physical stability of the enzyme particle, i.e. a low pressure drop corresponds to a good physical stability.

The experimental results (initial activity, half life and pressure drop) pre shown in Table 2.

Table 1 Molecular weight Polymer Mole % Mn K value

-CHrCH(NH,)- g/mol

A 67 4-5 x 105 106 B 100 4-5 x 105 106 C 32 2-3 x 10' 86 D 61 2-3 x 105 86 E 95 2-3 x 105 86 F 43 3-4 x 104 32 G 61 3-4 x 104 32 H 98 3-4 x 104 32 k NOD91/08287 PCr/DK90/00305 I- T a b0l a :2 Glucose isomerase Half life Pressure dr Polymer activity IGIU/g hours g/cm 2

Reference 350 2000 15 A 450 1000 50 B 300 2000 31 C 300 2200 11 D 350 2000 > 100 E 380 900 49 F 330 2000 26 G 340 1800 23 H 310 900 17 EXAMPLE 2 A series of preparation were made as in Example 1, except that the double amount of glutaraldehyde was used, i.e. 10 ml 50% glutaraldehyde before addition of polymer (or polyethyleneimine) and 20 ml after. The total amount of glutaraldehyde

thus amounts to 36%.

The results are shown in Table 3.

T Table 3 Polymer GI-activity IGIU/g Pressure drop, g/CM2 Sedipur 220 4 B 200 4 C 290 6 D 260 5 G 240 2 EXAMPLE 3 A series of preparations were made as in Example 1, except that half of the amount of polyethylene imine or the polymer of 1-amino ethylene with or without N-vinyl formamide (2.5% on dry matter). The results are shown in Table 4.

Table 4 Polymer GI-activity Half-life Pressure drop IGIU/g hours Sedipur 500 1100 148 B 480 - 184 C 550 1100 356 D 540 1200 551 G 505 - 127 0~ EXAMPLE 4 i.

1 liter of glucose isomerase containing fermentation liquid from Streptomyces murinus (4% dry substance content) was mixed with 10 g MgS04* 71-120 and pH adjusted to 7.5. 15 ml 50% glutaraldehyde was added and the cell sludge was stirred for 10 minutes under pH-adjustment to pH 7.5. Then 2.5% (on dry matter) of polymer C and 2.5% (on dry matter) of chitosan (Kayamic 400, a product from Nippon Kayaku Co., Ltd.) dissolved in 460 ml 0.5% acetic acid was added and thoroughly mixed. pH was added and thoroughly mixed. pH was constantly adjusted to 7.5. After 1 hour the cross-linked mixture was flocculated by addition of a cationic flocculant, Superfloc C521 (Cyanamid Int.). The cross-linked enzyme was recovered by filtration, formed into particles by extrusion through a 0.8 mm screen and dried at room temperature.

The results were:

GI-activity IGIU/g 450 Pressure drop 89 EXAMPI F 5 A series of preparations were made as in Example I except that all the glutaraldehyde was added to the fermentation liquid before the polymer and that the glutaraldehyde concentration was varied.

The results are shown in Table 5.

Table 5 -N S.

Polymer Glutaraldehyde GI-activity Half-life Pressure drop concentration, % IGIU/g hours g/cm, Sedipur 18 275 2000 4 Sedipur 27 240 1800 4 Sedipur 36 200 1500 7 C 18 300 1700 13 C 27 270 1500 20 C 36 205 1600 16 The results of the examples show that according to the invention, samples with good activity, stability and physical strength, suitable for use in fixed- bed column, can be obtained with various compositions of polymer and with various amounts of glutaraidehyde. Preparations with particularly good stability and physical strength are obtained with polymer containing 25-50% 1-amino ethylene.

Preparations with particularly good physical strength are obtained by using 30- % glutaraldehyde.

i X VOD91/08287 PC]r/DK90/00305 1.

Claims

CLAIMS 1. A particulate immobilized enzyme preparation obtainable by a process comprising the sequential steps of:

a) providing an aqueous medium containing enzymatically active biological material b) adding a polymer containing 1 -amino ethylene moieties and, optionally, N-vinyl formamide, c) adding a cross-linking agent for amino groups, d) holding the mixture to effect cross-linking and flocculation, e) dewatering, f) sub-dividing, and g) drying.

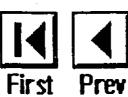
- 2. An immobilized preparation according to Claim 1, whereby the amount of said polymer is 2-30% by weight of the dry matter in the biological material, preferably 2-15%.
- 3. An immobilized preparation according to Claim 1 or 2, whereby said polymer contains 10-100 mole % (preferably 25-50%) of -CH2-CH(NH2)- units and 0-90% mole % (preferably 50~75%) of -CH2-CH (NH-CHO)- units, and preferably has a molecular weight of 50,000-500,000.
- 4. An immobilized preparation according to any preceding claim, whereby r chitosan is added prior to the intoduction of cross-linking agent in step c), preferably in an amount of 1-15% by weight of the dry matter in the biological material.
- 3; 5. An immobilized preparation according to any preceding claim, whereby said cross-linking agent is glutaraldehyde, polyazetidine or a diisocyanate.
- 6. An immobilized preparation according to any preceding CLAIMS obtainable by additionally adding cross-linking agent to the aqueous medium of step a) and -holding to effect partial cross-linking prior to the introduction of the polymer in step b), whereby the ratio of cross-linking agent added in step a) and step c) is preferably in the range 1:2 to 1:4.
- 7. An immobilized preparation according to any preceding claim, whereby the total amount of cross-linking agent used is 5-40% by weight of the dry matter in the biological material.
- 8. An immobilized preparation according to any preceding claim, whereby said biological material comprises dissolved enzyme, whole cells or cell mass from fully or partially disrupted cells, preferably of plant or microbial origin.
- 9. An immobilized preparation according to any preceding claim, whereby said biological material further comprises enzymatically inactive protein.
- 10. An immobilized preparation according to any preceding claim, whereby the biological material comprises glucose isomerase (preferably derived from a strain of Streptomyces, Bacillus or Actinoplanes), aminopeptidase (preferably derived from Pseudomonas), penicillin acylase (preferably from Fusarium), nitrilase (^) (preferably from Rhodococcus, Pseudomonas or Brevibacterium), fructosyl trans- ferase (preferably from Aspergillus), invertase (preferably from Saccharomyces), lactase (preferably from Kluyveromyces) or cyanidase (preferably from Alcali- genes).
- 3~11. Use (^) of an immobilized enzyme preparation according to any preceding
- CLAIMS in an enzyme-catalyzed process.
- 12. <u>Use (^)</u> according to the preceding claim in a continuous process with the immobilized enzyme in a fixed bed.
- 13. A process for immobilizing enzymatically active biological material, characterized by comprising the sequential steps of:
- a) providing an aqueous medium containing enzymatically active biological material b) adding a polymer containing 1 -amino ethylene moieties and, optionally, INTvinyl formamide, c) adding a cross-linking agent for amino groups, d) holding the mixture to effect cross-linking and flocculation, e) dewatering, f) sub-dividing, and g) drying.

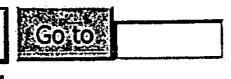


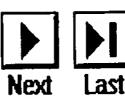












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